TECHNICAL COMMUNICATIONS

Supercritical Fluid Extraction/Enzyme Assay: A Novel Technique to Screen for Pesticide Residues in Meat Products

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The novel combination of supercritical fluid extraction (SFE) with an enzyme assay system has been used to screen meat products to detect the presence of pesticides. Analytes are collected in water by expanding supercritical carbon dioxide to atmospheric pressure through a restrictor and into an aqueous phase. The solution is then tested for the presence of pesticide residues by enzyme assay. Two experimental approaches have been used. Alachlor-fortified lard and bovine liver were monitored by static SFE coupled with an enzyme immunoassay. SFE of carbofuran-fortified frankfurters was coupled with an enzyme assay based on cholinesterase inhibition. A major benefit of the SFE/enzyme assay technique over conventional screening techniques is that the analyst is not exposed to organic solvents.

The increasing demand for monitoring pesticide residues in food products is accompanied by the need for novel analytical techniques that screen foods for the presence of pesticides. Such techniques can ultimately be developed into screening methods that determine whether a compound or class of compounds is present at some designated tolerance level in a meat matrix (1). In general, screening methods are useful because they allow a larger number of samples to be surveyed than could be accomplished with conventional analyses. Such screening methods are usually designed to detect the presence of an analyte but not necessarily the exact quantity of residue in the food matrix.

Enzyme assays, such as enzyme inhibition detection and enzyme-linked immunosorbent assays, are expected to play a prominent role in future screening methodologies (2). In fact, the number of immunoassays developed for use in trace pesticide analysis has continued to increase (3). However, one problem arises in attempting to integrate enzyme immunoassays with pesticide residue analyses. The classical methods for determining pesticide residues in fatty samples generally rely on organic solvent extractions to separate the compound(s) of interest from the sample matrix. Immunochemical assays, on the other hand, are conducted in aqueous media, although minor amounts of certain polar, water-miscible solvents can be tolerated (4). Organic solvents are known to disrupt the antigen-antibody reaction and can also denature the enzymes. Consequently, the integration of organic solvent-based extraction techniques with enzyme assays in a proposed screening method requires that additional steps be incorporated. For example, the organic solvent must be evaporated to dryness and the pesticide residues redissolved in an aqueous medium before the enzyme assay can be performed.

An alternative technique is to use supercritical fluid extraction (SFE) with carbon dioxide in place of organic solvents. Additional benefits can also be realized by using supercritical carbon dioxide (SC-CO_2) as the extraction solvent. Safety concerns associated with the use of organic solvents, such as waste disposal, flammability, and toxicity, are avoided.

SFE was recently investigated for use in pesticide residue analyses. For example, it was coupled with supercritical fluid chromatography to separate sulfonylurea herbicides and their metabolites from soil, cell culture media, and plant material (5). SFE was also used in conjunction with liquid chromatography, and it was shown to provide nearly quantitative recoveries for some pesticides (6). Organochlorine pesticides have been extracted from spiked lard (7) and fish tissue (8) by SFE.

In this communication, we describe 2 approaches to coupling SFE with enzyme assays. The purpose of this study was to demonstrate that SFE could be combined with enzyme assay methodology to yield a field-compatible technique for detecting the presence of pesticides in meat products. The described technique uses relatively nontoxic media (carbon dioxide, water) that allow it to be performed on-site in a food production facility. Because the extraction apparatus is very simple and inexpensive, it can be used by food inspectors at the plant level. No elaborate instrumentation or sample cleanup methods are required in this screening technique, and it can be used as a first step in determining whether violative levels of toxicants are present in a meat product.

Experimental

SC-CO_2 at moderate pressures (<151 atm) and temperatures (<69°C) is used to extract pesticides from fortified meat products. Analytes are solvated by SC-CO_2 and collected by expanding the pressurized CO_2 to atmospheric pressure through a restrictor directly into water in a collection vial. The aqueous solution is then tested for the presence of pesticides by an enzyme assay. Two experimental approaches were used. Alachlor-fortified lard and bovine liver were monitored by static SFE coupled with an enzyme immunoassay. In this approach, the sample is loaded into the extraction cell with dry ice. The sealed cell is then heated, which sublimes the dry ice, yielding SC-CO_2. In the second experimental approach, SFE of carbofuran-fortified frankfurters is coupled with an enzyme assay based on cholinesterase inhibition. In this instance, the extraction apparatus was equipped with a reservoir vessel filled with CO_2 from a storage cylinder with a dip tube.

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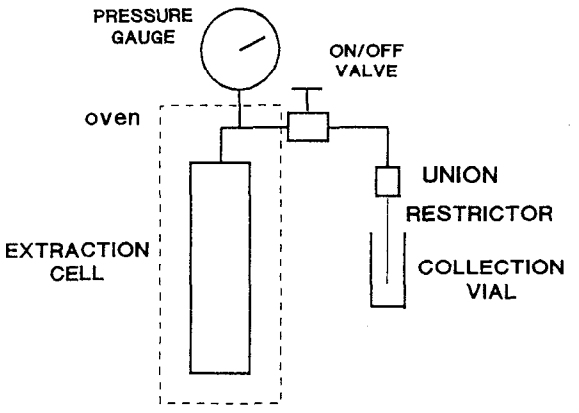

Figure 1. Schematic diagram of static SFE apparatus.

(a) *Pesticides.*—Alachlor and carbofuran (Pesticides and Industrial Chemicals Repository, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711).

(b) *Static SFE apparatus.*—Simple static extractions were performed with a 70 mL extraction vessel constructed of high pressure stainless steel tubing and fittings (Autoclave Engineers, Erie, PA 16512). One end of the extraction vessel was capped. The other end was connected to a pressure gauge and an exit line via a tee fitting, as schematically illustrated in Figure 1. Interconnecting lines consisted of 316 stainless steel tubing, 0.32 cm od, 0.159 cm id, having a more than adequate pressure rating. The extraction cell was positioned vertically in a Bendix gas chromatographic oven (Model 2600). The exit line was attached to a shut-off valve and connected to a length of PEEK tubing (Upchurch Scientific, Inc., Oak Harbor, WA 98277), 7 cm × 0.127 mm id, which served as a restrictor. The restrictor was vented into 5 mL deionized water held in 12 mL vial. The collection vial was placed in a beaker of water to moderate the cooling effect of the pressure drop. This step was required to prevent the water in the collection vial from solidifying into a mass of ice crystals. Dry ice (ca 40 g) was used to supply the CO_2 for SFE. Before the extraction cell was loaded, ca 40 g dry ice was used to initially cool the cell. This precaution limited CO_2 sublimation losses that occur before the cell is connected to the system.

(c) *Enzyme immunoassay (EIA).*—(Res-I-Quant, Alachlor Immunoassay Kit, Immunosystems, Scarborough, ME). Quantitative range of this assay is 0.5–20 ppb.

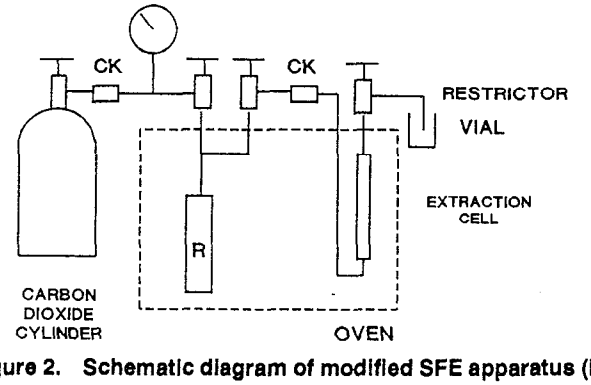

Figure 2. Schematic diagram of modified SFE apparatus (R = expansion reservoir, CK = check valve).

(d) *Microplate reader.*—Absorbances of the microtiter wells were measured with a microplate reader (Model EL-308, Bio-Tek Institutes, Winooski, VT).

(e) *Modified SFE apparatus.*—Extraction system was modified to enhance extraction efficiency to compensate for the decreased sensitivity exhibited by the enzyme inhibition assay. A schematic diagram of the modified SFE system is shown in Figure 2. The reservoir consisted of 316 stainless steel tubing (Part No. 15–009, Autoclave Engineers), 30.5 cm × 1.75 cm id. The extraction cell was 20.3 cm × 1.75 cm id. All other equipment is as cited in (a). To operate this system, the reservoir is cooled with an ice bath while being filled with CO_2 from a CO_2 cylinder equipped with a siphon tube. The head pressure (63 atm) of the cylinder is used to fill the reservoir. Concurrently, a sample is loaded into the extraction cell. Then, the appropriate valves are closed and the valve isolating the reservoir from the extraction cell is opened. The oven is heated to the extraction temperature and a supercritical fluid phase is produced. These extraction conditions are maintained for 15 min. CO_2 is then vented through the restrictor for ca 10 min until the pressure drops below the critical pressure (72.8 atm).

(f) *Enzyme inhibition assay.*—A commercial pesticide detection kit based on cholinesterase inhibition (Enzytec, Kansas City, KS) was also examined in conjunction with SFE. The assay can detect over 50 different pesticides, although it is not as sensitive as EIA. Carbofuran was detected in the collection water by a bioconcentration technique described by the manufacturer. In this approach, the detection limit for carbofuran in water is 0.003 ppm. Visual observation of the color of the test ticket indicated the presence or absence of the pesticide. The collection water for each sample was at room temperature for the enzyme assays.

(g) *Blender.*—Tissue samples were homogenized in a Model 1120 Waring blender (Waring Products Div., New Hartford, CT 06057) with dry ice (9).

(h) *Meat products.*—Lard samples were fortified with alachlor at levels up to 100 ppb, and they were supported on glass wool in the extraction cell. Frankfurter samples were fortified with carbofuran in amounts up to 0.3 ppm. Separate bovine liver samples were fortified with alachlor and carbofuran at 3.3 and 200 ppb, respectively.

Results and Discussion

Table 1 shows the screening results of the static SFE/EIA technique for some alachlor-fortified samples, using dry ice as the CO_2 source and the extraction apparatus as schematically illustrated in Figure 1. The technique confirmed the presence of alachlor in lard samples spiked at 10 ppb or greater. Blank extractions (no pesticide or sample) and negative control extractions (unspiked samples) yielded concentration values that in all cases were below those of the fortified samples. With the Student's paired *t*-test at a confidence limit of 95%, alachlor concentration in all of the spiked lard, except one sample spiked at 2.5 ppb, could be shown to be statistically greater than those in the negative control samples.

An estimate of the detection limit was determined for the alachlor EIA on the CO_2-bubbled water from the negative control and blank samples. The Shewhart chart (10) in Figure 3 graphically displays the results. The line at 1.1 ppb identifies the lower limit of detection. Samples yielding

Table 1. Screening results of static SFE/EIA on lard and bovine liver samples using SFE apparatus (Figure 1)

Sample	Alachlor detected[a]
Dry ice (× 3)	–
Lard (0.5–2.1 g), (× 3)	–
Alachlor (20 ng)	+
Lard (g), spiked with alachlor (ppb)	
1.9, 2.5	–
2.4, 10	+
2.5, 25	+
0.5, 50	+
1.1, 50	+
2.3, 50	+
0.5, 100	+
Bovine liver	
12 g, no spike	–
12 g, 3.3 ppb alachlor	+

[a] + = pesticide detected (concentration of alachlor in collection water is above detection limit); – = pesticide not detected (concentration of alachlor in water is below detection limit).

pesticide concentrations in the collection water below this limit cannot be statistically differentiated from unspiked samples. This detection limit was used to classify the samples into 1 of 2 categories; alachlor is present (+) or absent (–). Of course, the absolute detection threshold will depend on a number of factors, such as the sample mass in the extraction cell, the volume of collection water, and the SFE methodology used.

The described static extraction technique eliminated the need for a pump or compressor, thereby keeping the SFE system as simple and inexpensive as possible. Static SFE involves filling the extraction cell with the extracting fluid, allowing the fluid to equilibrate with the sample, and then depressurizing the fluid from the cell into a suitable collection solvent. Static SFE was shown to produce recoveries of 80–100% for organochlorine pesticides in fish (8). In our studies, dry ice was used to fill the extraction cell with CO_2. For the bovine liver samples, dry ice was also used as an aid in homogenizing the sample (9). In this manner, dry ice served 2 purposes. It kept tissue brittle, so that the blender could produce a fine homogenate, and it was also the source of CO_2 for SFE. The relatively low extraction pressure was chosen to minimize coextraction of lipids. Under the cited extraction conditions (>86 atm, 55°C, and extraction time 1.0–3.5 h), solubility of pure alachlor in SC-CO_2 is greater

Table 2. Screening results of SFE coupled with enzyme inhibition assay using modified SFE apparatus (Figure 2)

Spike level, ppm	Carbofuran detected[a]
Frankfurters (17 g)	
0.00	–
0.05	+
0.10	+
0.20	+
Bovine liver	
0.00	–
0.20	+

[a] + = enzyme ticket indicates presence of pesticide; – = enzyme ticket indicates absence of pesticide.

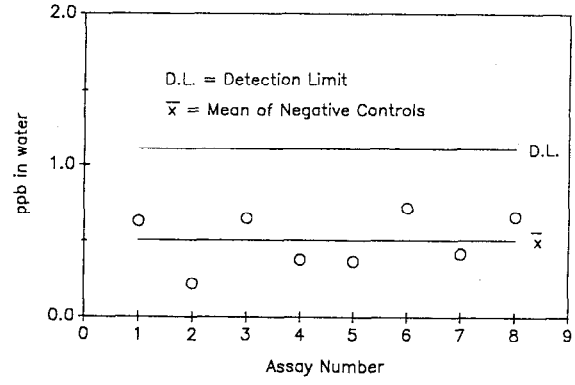

Figure 3. Shewhart chart of negative control collection water samples (enzyme immunoassay for alachlor, Res-I-Quant, Immunosystems).

than 0.01 wt% (11), sufficient for solubilizing trace amounts of alachlor.

A commercial pesticide residue detection system based on cholinesterase enzyme inhibition that is able to screen for a wide range of common pesticides (1) was also used in demonstrating the coupling of SFE and enzyme assay for pesticide residue screening. In general, the detection scheme used by this assay is not as sensitive as immunoassays. Hence, the static extraction system was modified to provide a more efficient sweeping of the extracting fluid from the extraction cell. Incorporation of a pump or compressor into the system was avoided to minimize equipment costs and to keep the SFE system simple. Similar approaches to fluid delivery have been reported (8, 12). The modified SFE system (Figure 2) supplied enough CO_2 to flush the volume of the extraction cell at least 3 times. Results from the SFE/enzyme inhibition assay on frankfurter and bovine liver samples spiked with carbofuran are given in Table 2 (extraction conditions: 150–72 atm and 68°C for 45 min.). For each spiked sample, a negative control sample was also extracted for comparison. The color of the ticket from the negative control sample was then compared to that of the spiked sample. Spiked samples were identified as having more of the enzyme inhibited than the negative controls.

In conclusion, several benefits of the SFE/enzyme assay technique are worth noting. The selective nature of enzyme assays allow inexpensive welding-grade CO_2 or dry ice to be used in screening for trace residues. The use of water and CO_2 avoids the need for any organic solvents in the extraction, concentration, or detection steps. The described technique also reduces the number of steps needed to perform enzyme assays for pesticide residue detection. The coupled technique was able to distinguish samples spiked at levels close to the tolerance limit for residues in meats (alachlor = 0.020 ppm; carbofuran = 0.050 ppm) (13). Further development of this screening technique for other food matrixes and toxicants will be forthcoming.

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